

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|--|---|
| (51) International Patent Classification 6 : C12N 15/86 | A1 | (11) International Publication Number: WO 98/04727 (43) International Publication Date: 5 February 1998 (05.02.98) |
| (21) International Application Number: PCT/US97/12203 | (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). | |
| (22) International Filing Date: 15 July 1997 (15.07.97) | | |
| (30) Priority Data: 08/686,280 25 July 1996 (25.07.96) US | Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> | |
| (71) Applicants: THERION BIOLOGICS CORPORATION [US/US]; 76 Rogers Street, Cambridge, MA 02142 (US). UNITED STATES GOVERNMENT, as represented by THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US). | | |
| (72) Inventors: SCHLOM, Jeffrey; 10301 Sorrel Avenue, Potomac, MD 20854 (US). PANICALI, Dennis, L.; 114 Nonset Path, Acton, MA 02139 (US). GRITZ, Linda, R.; 3 Emerson Street Somerville, MA 02143 (US). MAZZARA, Gail, P.; 22 Rangeley Road, Winchester, MA 01890 (US). | | |
| (74) Agents: BRONSTEIN, Sewall, P. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109 (US). | | |

(54) Title: RECOMBINANT POX VIRUS FOR IMMUNIZATION AGAINST TUMOR-ASSOCIATED ANTIGENS

(57) Abstract

Recombinant pox viruses capable of expressing cell-encoded, tumor-associated antigens are disclosed. The recombinant viruses are useful for evoking an immune response against the antigen.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|----|--------------------------|----|---------------------------------------|----|---|----|--------------------------|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | ML | Mali | TR | Turkey |
| BG | Bulgaria | HU | Hungary | MN | Mongolia | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MR | Mauritania | UA | Ukraine |
| BR | Brazil | IL | Israel | MW | Malawi | UG | Uganda |
| BY | Belarus | IS | Iceland | MX | Mexico | US | United States of America |
| CA | Canada | IT | Italy | NE | Niger | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NL | Netherlands | VN | Viet Nam |
| CG | Congo | KE | Kenya | NO | Norway | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NZ | New Zealand | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | PL | Poland | | |
| CM | Cameroon | KR | Republic of Korea | PT | Portugal | | |
| CN | China | KZ | Kazakhstan | RO | Romania | | |
| CU | Cuba | LC | Saint Lucia | RU | Russian Federation | | |
| CZ | Czech Republic | LI | Liechtenstein | SD | Sudan | | |
| DE | Germany | LK | Sri Lanka | SE | Sweden | | |
| DK | Denmark | LR | Liberia | SG | Singapore | | |
| EE | Estonia | | | | | | |

RECOMBINANT POX VIRUS FOR IMMUNIZATION AGAINST TUMOR-ASSOCIATED ANTIGENS

Background of the Invention

The immunotherapeutic approach to the treatment of cancer is based on the observation that human tumor cells express a variety of tumor-associated antigens (TAAs) that are not expressed in normal tissues. These antigens, which include viral tumor antigens, cellular oncogene proteins, and tumor-associated differentiation antigens, can serve as targets for the host immune system and elicit responses which result in tumor destruction. This immune response is mediated primarily by lymphocytes; T cells in general and class I MHC-restricted cytotoxic T lymphocytes in particular play a central role in tumor rejection. Hellstrom, K.E., et al., (1969) *Adv. Cancer Res.* 12:167-223; Greenberg, P.D. (1991) in *Advances in Immunology*, vol. 49 (Dixon, D.J., ed.), pp 281-355, Academic Press, Inc., Orlando, FL. Unfortunately, as evidenced by the high incidence of cancer in the population, the immune response to neoplastic cells often fails to eliminate tumors. The goal of active cancer immunotherapy is the augmentation of anti-tumor responses, particularly T cell responses, in order to effect complete tumor destruction.

Most attempts at active immunization against cancer antigens have involved whole tumor cells or tumor cell fragments. However, the cloning of TAAs recognized by CD8+ T cells has opened new possibilities for the immunotherapy of cancer based on the use of recombinant or synthetic anti-cancer vaccines. Boon, T., et al.,(1994) *Annu. Rev. Immunol.* 12:337-365; Brithcard, V., et al., (1993) *J. Exp. Med.* 178:489-495; Cox, A.L., et al., (1994) *Science* 264:716-719;

- 2 -

Houghton, A.N. (1994) J. Exp. Med. 180:1-4; Pardoll, D.M. (1994) Nature 369:357-358; Kawakami, Y., et al., (1994) Proc. Natl. Acad. Sci. U.S.A. 91:3515-3519; Kawakami, Y., et al., (1994) Proc. Natl. Acad. Sci. U.S.A. 91:6458-6462.

5

Two such antigens have been designated MART-1 (Melanoma Antigen Recognized by T cells - 1) and gp100. *Proc. Natl. Acad. Sci. U.S.A.* 91:3515-3519. MART-1 and gp 100 appear to be expressed in virtually all fresh and cultured melanomas. With the exception of 10 melanocyte and retina, no normal tissues express the antigens. The antigens may be responsible for mediating tumor regression in patients with advanced melanoma, since the tumor-infiltrating lymphocytes (TIL) used to identify MART-1 and gp100 were capable of effecting tumor regression *in vivo*. Thus, immunization of melanoma patients with 15 MART-1 or gp100 may boost their cellular immune responses against their cancers.

The use of recombinant vaccinia viruses for anti-tumor immunotherapy has been reviewed. (Hu, S.L., Hellstrom, I., and 20 Hellstrom K.E. (1992) in *Vaccines: New Approaches to Immunological Problems* (R.W. Ellis, ed) pp 327-343, Butterworth-Heinemann, Boston.) Anti-tumor responses have been elicited using a recombinant vaccinia virus expressing a TAA designated carcinoembryonic antigen (CEA). CEA is a glycoprotein expressed at high level on the surface of 25 nearly all tumors of the gastrointestinal tract, as well as on many mammary carcinomas and lung adenocarcinomas. (Muraro, R., et al., (1985) *Cancer Res.* 45:5769-5780.) A recombinant vaccinia virus that expresses CEA (Kantor, J., et al. (1992) *J. Natl. Cancer Inst.* 84:1084-1091) was evaluated using a murine tumor model in which 30 the human CEA gene was transduced into murine colon carcinoma

- 3 -

cells. (Robbins, P.F., et al. (1991) *Cancer Res.* 51:3657-3662.) Mice immunized with the CEA/vaccinia recombinant were resistant to the growth of subsequently transplanted CEA-expressing tumors.
Moreover, when mice bearing established CEA-transduced murine
5 carcinomas were treated with the recombinant virus, the tumors showed greatly reduced growth or complete regression. In rhesus monkeys, which carry an antigen on the surface of their granulocytes that cross-reacts with human CEA, immunization with the recombinant elicited anti-CEA antibodies, delayed type hypersensitivity, and
10 lymphoproliferative responses. (Kantor, J., et al. (1992) *Cancer Res.* 52:6917-6925.) No toxicity was observed.

Prostate-specific antigen (PSA) is a 33,000-34,000 dalton glycoprotein that is produced in normal, benign, and cancerous prostate epithelia, but not in other normal or malignant tissues. (Wang, M.C., et
15 al. (1982) *Meth Cancer Res.* 19):179-197.) PSA is secreted into prostatic fluid and seminal plasma. (See, Wang, et al.) Elevation of PSA levels in serum is correlated with growth of the prostate, and prostate cancer patients show an exponential increase in PSA levels.
20 (Carter, H.B., et al. (1992) *Cancer Research* 52:3323-3328.) Due to its tissue specificity, PSA is a potential target antigen for immunotherapy against prostate cancer.

A number of laboratories have explored the use of recombinant
25 poxviruses that express specific TAAs as immunotherapeutic vaccines. The ability of recombinant poxviruses expressing a variety of antigens to serve as potential vaccines for the prevention of infectious disease has been well-documented. Immunization with live recombinant pox virus allows expression of foreign antigens that are presented to the
30 immune system together with highly immunogenic, virus proteins,

- 4 -

which may act as adjuvants to enhance immune responses to the foreign antigen. Austin, F.C., et al. (1979) *Adv. Cancer Res.* 30:301-345. Finally, poxviruses are not oncogenic and do not integrate into the host cell genome, as replication and transcription of genetic material occurs in the cytoplasm of the infected cell.

Viruses of the family *Poxviridae* (pox viruses) are useful as vectors for the delivery of foreign genes and gene products in many clinical and research settings. Pox viruses of the genus *Orthopoxvirus*, particularly vaccinia, are used for several reasons. Among these are:

(a) its wide use in humans in the eradication of smallpox; (b) its ability to infect a wide range of cells, including professional antigen presenting cells, and express the inserted gene product (i.e. foreign gene product) in a manner that has the potential to be processed in the context of class I and/or class II MHC molecules; and (c) use as a recombinant vaccine in the treatment of certain tumors (Kantor, J. et al. (1992)).

Fowlpox virus (FPV) is a member of the avipox virus family. Productive FPV infection is restricted *in vivo* to cells derived from avian species; however, FPV-mediated gene expression does occur in infected non-avian cells. Taylor, J. et al., (1988) *Vaccine* 6:497-503. Fowlpox virus based recombinant vaccines are described in: *Technological Advances in Vaccine Development* (Alan R. Liss, Inc.) pp. 321-334. Furthermore, *in vivo* FPV-mediated gene expression in several mammalian species has been demonstrated. Six non-avian species immunized with live recombinant fowlpox virus expressing the rabies glycoprotein developed antibodies against this glycoprotein. Immunization with this recombinant FPV elicited antibodies against this glycoprotein. Immunization with this recombinant FPV partially protected mice, cats, and dogs against a rabies virus challenge. There

- 5 -

was no manifestation of proliferative infection or overt disease in any animals immunized with a variety of doses of live recombinant FPV.

Taylor, J., et al., (1988) Vaccine 6:497-503. In another study, a recombinant FPV containing a measles fusion protein was shown to 5 partially protect mice against lethal challenge the measles virus, although antibodies against the fusion protein were not detected. Wild, T.F., et al., (1990) Vaccine 8:441-442. It was therefore postulated that protection was mediated by cellular immune responses. These results suggest that recombinant FPV may have utility as a safe and 10 effective alternative to vaccinia virus as a vaccine vector.

Summary of the Invention

The present invention relates to recombinant pox viruses capable of expressing cell-encoded tumor associated antigens and/or 15 immunomodulators.

Recombinant pox virus capable of expressing a cell-encoded tumor-associated antigen are produced by integrating into the pox virus genome sequences encoding the antigen or immunogenic portions 20 thereof. Tumor associated antigens include molecules expressed by tumor cells (e.g. carcinoembryonic antigen, prostate-specific antigen (PSA), MUCIN (MUC-1), melanoma associated antigens such as MART-1, etc.). Immunomodulators include interleukin 2, B7.1 and B7.2.

25 Particularly preferred recombinant pox viruses include TBC-CEA (vaccinia, CEA), PROSTVAC (vaccinia, PSA), rV-B7.1 (vaccinia, B7), rF-B7.1 (fowlpox, 7.1), rF-CEA (fowlpox, CEA), rV-MUC-1 (vaccinia, MUC-1) and rF-PSA (fowlpox, PSA).

30 Brief Description of the Figures

- 6 -

Figure 1 is a plasmid map of pT1001, the donor plasmid used in
the construction of PROSTVAC.

5 Figure 2 is a plasmid map of pT108, the donor plasmid used in
the construction of TBC-CEA.

Figure 3 is a plasmid map of pT109, the donor plasmid used in
the construction of rF-CEA.

10 Figure 4 is a plasmid map of pT1046, the donor plasmid used in
the construction of rV-B7.1.

Figure 5 is a plasmid map of pT1058, the donor plasmid used in
the construction of rF-B7.1.

15 Figure 6 is a plasmid map of pT2078, the donor plasmid used in
the construction of rF-PSA.

20 Figure 7 is a plasmid map of pT2068, the donor plasmid used in
the construction of rV-muc-1.

Detailed Description of the Invention

Pox viruses serve as effective vectors for inducing immunity
against tumor-associated antigens.

25 Particularly preferred tumor-associated antigens are cell surface
molecules. These are positioned for recognition by elements of the
immune systems and thus are excellent targets for immunotherapy.

30 Tumor-associated antigens are expressed by certain tumor cells

- 7 -

and provide effective targets for immunotherapy. Some examples are carcinoembryonic antigen (CEA) and prostate-specific antigen (PSA).

5 Immunomodulators can regulate immune responses, increasing the likelihood of a sufficient cellular immune response occurring, (e.g. IL-2) or can provide cellular ligand necessary for stimulating a CTL response (e.g. B7.1 or B7.2)

10 Basic techniques for preparing pox viruses containing a heterologous DNA sequence encoding the tumor-associated antigen known to the skilled artisan and involve, for example, homologous recombination between the viral DNA sequences flanking the DNA sequence in a donor plasmid and homologous sequences present in the parental virus (Mackett, et al., *Proc. Natl. Acad. Sci. USA* 79:7415-7419 (1982)). For example, recombinant viral vectors such as a pox viral vector can be used in delivering the gene. The vector can be constructed for example by steps known in the art, e.g. analogous to the methods for creating synthetic recombinants of the fowlpox virus described in U.S. Patent No. 5,093,258, the disclosure of which is incorporated herein by reference. Other techniques include using a unique restriction endonuclease site that is naturally present or artificially inserted in the parental viral vector to insert the heterologous DNA. See, U.S. Patent No. 5,445,953, incorporated herein by reference.

15 20 25

Pox viruses useful in practicing the present invention include orthopox, suipox, avipox and capripoxvirus.

Orthopox virus include vaccinia, ectromelia and raccoon pox.
30 The preferred orthopox is vaccinia. More preferred is a sub-clone of

- 8 -

vaccinia having decreased virulence relative to a standard vaccine strain of vaccinia.

5 Avipox viruses include fowlpox, canary pox and pigeon pox. The preferred avipox is fowlpox.

A preferred suipox is swinepox.

10 For example, the DNA gene sequence to be inserted into the virus can be placed into a donor plasmid, e.g., an *E. coli* plasmid construct. Separately the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA which is the desired insertion region. With a parental pox viral vector, 15 a pox promoter is used. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria and isolated. Preferably, the plasmid also contains an origin of replication such as the *E. coli* origin of replication, and a marker such as an antibiotic resistance gene for 20 selection and propagation in *E. coli*.

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts, along with the parental virus, e.g., poxvirus.

25 Recombination between homologous pox DNA in the plasmid and the viral genome respectively results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome, at a site which does not affect virus viability.

30 As noted above, the gene is inserted into a region (insertion

- 9 -

region), in the virus which does not affect virus viability of the resultant recombinant virus. The skilled artisan can readily identify such regions in a virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without seriously affecting 5 virus viability of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene. For example, the TK gene has been found in all pox virus genomes examined [leporipoxvirus: Upton, et al., *J. Virology*, 60:920 (1986) (shope fibroma virus); capripoxvirus: Gershon, et al., *J. Gen. Virol.*, 10 70:525 (1989) (Kenya sheep-1); orthopoxvirus: Weir, et al., *J. Virol.*, 46:530 (1983) (vaccinia); Esposito, et al., *Virology*, 135:561 (1984) (monkeypox and variola virus); Hruby, et al., *PNAS*, 80:3411 (1983) (vaccinia); Kilpatrick, et al., *Virology*, 143:399 (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al., *J. Gen. Virol.* 69:1275 (1988) 15 (fowlpox); Boyle, et al., *Virology*, 156:355 (1987) (fowlpox); Schnitzlein, et al., *J. Virological Methods*, 20:341 (1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., *J. Gen. Virol.* 73:3235-3240 (1992)].

20 In vaccinia, in addition to the TK region, other insertion regions include, for example, HindIII M.

In fowlpox, in addition to the TK region, other insertion regions include, for example, BamHI J [Jenkins, et al., *AIDS Research and 25 Human Retroviruses* 7:991-998 (1991)] the EcoRI-HindIII fragment, BamHI fragment, EcoRV-HindIII fragment, BamHI fragment and the HindIII fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, et al., *J. of Virol.* 67:3069-3076 (1993); Taylor, et al., Vaccine 6:497-503 (1988); Spehner, et al., (1990) and Boursnell, et 30 al., *J. of Gen. Virol.* 71:621-628 (1990)].

- 10 -

In swinepox preferred insertion sites include the thymidine kinase gene region.

5 In addition to the requirement that the gene be inserted into an insertion region, successful expression of the inserted gene by the modified poxvirus requires the presence of a promoter operably linked to the desired gene, i.e., in the proper relationship to the inserted gene. The promoter must be placed so that it is located upstream from the
10 gene to be expressed. Promoters are well known in the art and can readily be selected depending on the host and the cell type you wish to target. For example in poxviruses, pox viral promoters should be used, such as the vaccinia 7.5K, or 40K or fowlpox C1. Artificial constructs containing appropriate pox sequences can also be used. Enhancer
15 elements can also be used in combination to increase the level of expression. Furthermore, the use of inducible promoters, which are also well known in the art, are preferred in some embodiments.

Vaccines

20 Live recombinant viruses expressing an immunogenic cell encoded tumor associated antigen can be used to induce an immune response against tumor cells which express the protein. These recombinant viruses may be administered by intradermal scarification, as was conventionally done for small pox vaccination, or by other
25 routes appropriate to the recombinant virus used. These may include among others, intramuscular, subcutaneous, and intravenous routes. Vaccination of a host organism with live recombinant vaccinia virus is followed by replication of the virus within the host.

30 For parenteral administration, the recombinant vectors will

- 11 -

typically be injected in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable carrier such as physiological saline.

5 A specific immune response to a tumor associated antigen can
be generated by administering between about 10⁵-10⁹ pfu of the
recombinant pox virus, constructed as discussed above to a host, more
preferably one uses $\geq 10^7$ pfu. The preferred host is a human. At least
one interval thereafter, which is preferably one to three months later,
10 the immune response is boosted by administering additional antigen to
the host. More preferably there is at least a second "boost" preferably
at least one to three months after the first boost, more preferably 6-12
months after the first boost. The antigen for boosting may be
administered using the same pox virus vector. The boosting antigen
15 may be administered as a whole protein, an immunogenic peptide
fraction of the protein, or DNA encoding the protein or peptide. The
boosting antigen may preferably be administered using a second pox
virus vector from a different pox genus, or may be administered
directly, for example, purified protein plus an adjuvant or in a liposome
20 formation. Cytokines, e.g., IL-2, IL-6, IL-12, IL-15, or co-stimulatory
molecules, e.g., B7.1, B7.2, may be used as biologic adjuvants. The
cytokines can be administered systemically to the host, either cytokines
or costimulatory molecules can be co-administered via insertion of the
genes encoding the molecules into the recombinant pox vector or a
25 second recombinant poxvirus which is admixed with the recombinant
poxvirus expressing the TAA.

30 Adjuvants include, for example, RIBI Detox (Ribi
Immunochemical), QS21 (Cambridge Biotech), incomplete Freund's
adjuvant or many others.

- 12 -

Generation of Cytotoxic T-Cells

Cytotoxic T-cells specific for a tumor specific antigen can be established from peripheral blood mononuclear cells (PBMC) obtained from a host immunized as discussed above. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described [Boyum, et al., *Scand J. Clin Lab Invest* 21: 77-80 (1968)]. Washed PBMC are resuspended in a complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freeze Clinical System, Brown Dear, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100 µg/ml of streptomycin (GIBCO). PBMC at a concentration of about 2×10^5 cells in complete medium in a volume of, for example, 100 µl are added into each well of a 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). The antigen or peptides are added into the cultures in a final concentration of about 50 µg/ml and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 5 days. After removal of peptide containing media, the cultures are provided with fresh human IL-2 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the same peptide (50 µg/ml) on day 16. 5×10^5 irradiated (4,000 rad) autologous PBMC are added in a volume of about 50 µl complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with human IL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

- 13 -

Cell Therapy

The cytotoxic T-cell can be cultured to amplify its number and then injected back into the host by a variety of means. Generally, between 1×10^5 and 2×10^{11} cytotoxic T-cells per infusion are 5 administered in, for example, one to three infusions of 200 to 250 ml each over a period of 30 to 60 minutes. After the completion of the infusions, the patient may be treated with recombinant interleukin-2 with a dose of 720,000 IU per kilogram of body weight intravenously 10 every eight hours; some doses can be omitted depending on the patient's tolerance for the drug. In addition, after infusion, additional antigen or fragments containing T-cell eliciting epitope(s) may be administered to the patient to further expand the T-cell number. The antigen or epitope may be formulated with an adjuvant and/or may be 15 in a liposomal formulation.

15

The cytotoxic T-cells can also be modified by introduction of a viral vector containing a DNA encoding TNF and reintroduced into a host in an effort to enhance the anti-tumor activity of the cells. Other cytokines can also be used.

20

REFERENCE EXAMPLE 1 CONSTRUCTION OF VECTORS

25

Pox Viruses

A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., *Cell* 37:1053-1062 (1984); Morin et al., *Proc. Natl. Acad. Sci. USA*, 30 84:4626-4630 (1987); Lowe et al., *Proc. Natl. Acad. Sci. USA*, 84:3896-3900 (1987); Panicali & Paoletti, *Proc. Natl. Acad. Sci. USA*,

- 14 -

79:4927-4931 (1982); Machett et al., *Proc. Natl. Acad. Sci. USA*,
79:7415-7419 (1982)). Representative fowlpox and swinepox virus
are available through the ATCC under accession numbers VR-229 and
VR-363, respectively. The Wyeth strain of vaccinia is available through
5 the ATCC under accession number VR-325.

The parental fowlpox virus is a plaque purified isolate of the
POXVAC-TC vaccine strain of fowlpox virus (Schering Corp.).

10 The parental vaccinia virus (clone B-3-1) is a plaque-purified
isolate of the Wyeth strain that was received from Flow Laboratories.

15 To obtain clone B-3-1, the Wyeth strain of vaccinia was
passaged in the fetal rhesus lung line FRhL (ATCC Accession No.
CL160) as follows:

Pass 1: Plaque "B" was picked at $10^{-5.3}$ dilution
Pass 2: Plaque "B-3" was picked at $10^{-1.6}$ dilution
Pass 3: Plaque "B-3-1" was picked at $10^{-1.6}$ dilution
20 Pass 4-7: Plaque B-3-1 was serially passaged using serum-free
medium with sucrose phosphate glutamate (SPG) to
prepare a small seed pool at the 7th passage level.

25 The virulence of this plaque isolate of the Wyeth vaccinia strain
was assessed by determining the infections dose of the virus lethal to
50% (LD_{50}) of weaning mice infected intracranially. Two to three week
old immunocompetent mice were inoculated with various doses of virus
(7 mice/dose); the LD_{50} was determined on mice succubing between 2
and 12 days post-inoculation by calculating the 50% endpoint using
30 the Reed-Muench method (20). These values were compared to those

- 15 -

obtained using a virus stock prepared by expanding virus directly from a vial of the CDC Smallpox Vaccine (Table 1).

Table 1

5

Neurovirulence of Wyeth Vaccinia Virus in Mice

| Experiment # | LD ₅₀ (plaque forming units) | |
|-----------------|---|-------------------|
| | Wyeth master virus stock | Small pox vaccine |
| 1 | ND* | 10 ^{5.4} |
| 2 | ND | 10 ^{5.6} |
| 3 | 10 ^{6.7} | > 10 ⁶ |
| 4 | 10 ^{6.8} | 10 ^{5.4} |
| 5 | 10 ^{7.3} | ND* |
| 6 | 10 ^{7.7} | ND |
| 7 | 10 ^{7.3} | 10 ^{5.7} |

* not determined

DNA Vectors For *In Vivo* Recombination With A Parent Virus

Genes that code for desired carcinoma associated antigens are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor vector for *in vivo* recombination with a pox virus.

15

In general, the DNA donor vector contains the following elements:

- (i) a prokaryotic origin of replication, so that the vector may

- 16 -

be amplified in a prokaryotic host;

(ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);

5 (iii) at least one gene encoding a desired protein located adjacent to a transcriptional promoter capable of directing the expression of the gene; and

(iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

10

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in WO91/19803, the techniques of which are incorporated herein by reference. In general, 15 all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The donor plasmids can 20 be mono-, di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

The donor vector preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses 25 containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al., *J. Virol.*, 62:1046 (1988); Falkner and Moss., *J. Virol.*, 62:1849 (1988); Franke et al., *Mol. Cell. Biol.*, 5:1918 30 (1985), as well as genes such as the *E. coli lacZ* gene, that permit

- 17 -

identification of recombinant viral plaques by colorimetric assay
(Panicali et al., *Gene*, 47:193-199 (1986)).

5 Integration Of Foreign DNA Sequences Into The
Viral Genome And Isolation Of Recombinants

Homologous recombination between donor plasmid DNA and
viral DNA in an infected cell results in the formation of recombinant
viruses that incorporate the desired elements. Appropriate host cells
for *in vivo* recombination are generally eukaryotic cells that can be
10 infected by the virus and transfected by the plasmid vector. Examples
of such cells suitable for use with a pox virus are chick embryo dermal
(CED) cells, HuTK143 (human) cells, and CV-1 and BSC-40 (both
monkey kidney) cells. Infection of cells with pox virus and transfection
of these cells with plasmid vectors is accomplished by techniques
15 standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112,
WO89/03429).

Following *in vivo* recombination, recombinant viral progeny can
be identified by one of several techniques. For example, if the DNA
20 donor vector is designed to insert foreign genes into the parent virus
thymidine kinase (TK) gene, viruses containing integrated DNA will be
TK⁻ and can be selected on this basis (Mackett et al., *Proc. Natl. Acad.
Sci. USA*, 79:7415 (1982)). Alternatively, co-integration of a gene
encoding a marker or indicator gene with the foreign gene(s) of
25 interest, as described above, can be used to identify recombinant
progeny. One preferred indicator gene is the *E. coli lacZ* gene:
recombinant viruses expressing β-galactosidase can be selected using a
chromogenic substrate for the enzyme (Panicali et al., *Gene*, 47:193
(1986)).

- 18 -

Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. The presence of integrated foreign DNA can be detected by hybridization with a labeled DNA probe specific for the inserted DNA. Preferred techniques for selection, 5 however, are based upon co-integration of a gene encoding a marker or indicator gene along with the gene of interest, as described above. A preferred indicator gene is the *E. coli lacZ* gene which encodes the enzyme β-galactosidase. Selection of recombinant virus expressing β-galactosidase can be done by employing a chromogenic substrate for 10 the enzyme. For example, recombinant viruses are detected as blue plaques in the presence of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside or other halogenated-indolyl-β-D-galactoside (e.g., Bluogal™).

15 **Characterizing The Viral Antigens Expressed By Recombinant Viruses**

Once a recombinant virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), 20 Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme immunoassay (EIA).

EXAMPLE 1

Construction of Recombinant Vaccinia Virus (PROSTVAC)

25 A 786 bp DNA fragment encoding the entire open reading frame of human prostate specific antigen was amplified by reverse transcriptase PCR (GeneAmp RNA PCR Kit, Perkin Elmer, Norwalk, CT) from total RNA extracted from the human metastatic prostate adenocarcinoma cell line, LNCaP.FGC (CRL 1740, American Type 30 Culture Collection (ATCC), Rockville, MD). The predicted amino acid

- 19 -

sequence derived from the PSA coding sequence was shown to be nearly identical to the published sequence (Lundwall, et al., *FEBS Letters*, 214:317-322, (1987), differing only in a change from asparagine to tyrosine at position 220. The PSA DNA fragment, 5 containing the entire coding sequence for PSA, 41 nucleotides of the 5' untranslated region, and 520 nucleotides of the 3' untranslated region, was inserted into the Xba I restriction endonuclease cleavage site of the vaccinia virus transfer vector pT116. The resulting plasmid, 10 designated pT1001, contains the PSA gene under the control of the vaccinia virus 40K promoter (Gritz, et al., *J. Virol.* 64:5948-5957, (1990), and the *E. coli lacZ* gene under the control of the fowlpox virus C1 promoter (Jenkins, et al., *AIDS Research and Human Retroviruses* 7:991-998, (1991). The foreign genes are flanked by DNA sequences 15 from the Hind III M region of the vaccinia genome. Figure 1 is a plasmid map of PT1001.

A plaque-purified isolate from the Wyeth (New York City Board of Health) strain of vaccinia was used as the parental virus in the construction of the recombinant vaccinia virus, PROSTVAC. The 20 generation of recombinant vaccinia virus was accomplished via homologous recombination between vaccinia sequences in the Wyeth vaccinia genome and the corresponding sequences in pT1001 in vaccinia-infected RK₁₃ cells (CCL 37, ATCC) transfected with pT1001. Recombinant virus was identified using a chromogenic assay, 25 performed on viral plaques *in situ*, that detects expression of the *lacZ* gene product in the presence of halogenated indolyl-beta-D-galactoside (Bluo-gal), as described previously (Panacali, et al., 1986). Appropriate blue recombinant viruses were purified by four rounds of plaque-purification. Virus stocks were prepared by clarifying infected RK₁₃ cell 30 lysates followed by centrifugation through a 36% sucrose cushion.

- 20 -

EXAMPLE II

Construction of Recombinant Fowlpox Virus (rF-CEA)

The generation of recombinant fowlpox viruses is accomplished
5 via homologous recombination *in vivo* between fowlpox DNA and a
plasmid vector that carries the heterologous sequences to be inserted.
The plasmid vectors contain one or more chimeric genes, each
comprising a poxvirus promoter linked to a protein coding sequence,
flanked by viral sequences from a non-essential region of the fowlpox
10 virus genome. The plasmid is transfected into cells infected with the
parental fowlpox virus, and recombination between fowlpox sequences
on the plasmid and the corresponding DNA in the viral genome results
in the insertion into the viral genome of the chimeric genes on the
plasmid.

15

The plasmid vector (pT109) used for insertion of the CEA gene
into the parental fowlpox virus genome by *in vivo* recombination is
illustrated in Figure 3. This vector contains the following elements: (1)
a prokaryotic origin of replication to allow amplification of the vector in
20 a bacterial host; (2) the gene encoding resistance to the antibiotic
ampicillin, to permit selection of prokaryotic host cells that contain the
plasmid; (3) DNA sequences homologous to the BamHIJ region of the
fowlpox genome, which direct insertion of foreign sequences into this
region via homologous recombination; (4) a chimeric gene comprising
25 the vaccinia 40K transcriptional promotor linked to the CEA gene; (5) a
second chimeric gene comprising the fowlpox C1 transcriptional
promoter linked to the *E. coli lacZ* gene.

30 The gene encoding CEA was isolated at the National Cancer
Institute from a cDNA library derived from RNA from a human colon

- 21 -

carcinoma cell line as disclosed by Kaufman et al., (1991) *Int. J. Cancer* 48, 900-907, the disclosure of which is herein incorporated by reference.

5 A plaque-purified isolate from the POXVAC-TC vaccine strain of fowlpox virus was used as the parental virus for this recombinant vaccine. *In vivo* recombination between the plasmid vector and the viral DNA resulted in the formation of a recombinant virus in which the CEA gene, under the transcriptional direction of the vaccinia 40K promoter, and the *lacZ* gene, under the control of the C1 promoter, were inserted into the BamHIJ region of the fowlpox virus genome.

10 A chromogenic assay for β -galactosidase was used to identify recombinant viruses containing the *lacZ* and CEA sequences. This method takes advantage of the ability of fowlpox virus to form distinct plaques when grown on monolayers of CED cells. After *in vivo* recombination, cells were infected with progeny virus until distinct plaques were visible, at which time the plaques were overlaid with a chromogenic substrate for Beta-galactosidase (Bluo-gal). Viral plaques expressing *lacZ* appeared blue against a clear background. Positive plaques were picked from the cell monolayer and their progeny were further propagated. Repeated rounds of plaque isolation and replating in a presence of Bluo-gal resulted in the purification of the desired recombinant. Positive recombinants were amplified to produce a seed stock on CED cells. The seed stock was then subjected to titration, genomic and protein expression analysis.

15 The structure of the plasmid transfer vector was verified by restriction endonuclease digestion using Xba I and BamHI. In addition, the products of digestion with these enzymes were subjected to

- 22 -

Southern blot analysis using labeled probes corresponding to the CEA gene and to the fowlpox BamHI J sequences. The DNA fragments visualized by these methods were of the predicted sizes, and the presence of the CEA gene was unequivocally demonstrated, thus confirming the predicted structure of the plasmid.

5

The recombinant pox virus set forth below in Table 2 can be constructed using similar techniques.

10

TABLE 2

| RECOMBINANT POX* | DONOR PLASMID | TUMOR ASSOCIATED GENE/IMMUNO-MODULATOR | REFERENCE FOR GENE |
|--------------------|------------------|--|---|
| TBC-CEA rF-CEA | pT108 pT109 | CEA | Beauchemin et al., Mol. Cell. Biol. 7:3221-3230 |
| rV-B7.1 rF-B7.1 | pT1046 pT1058 | B7.1 | Freeman et al., 1989, J. Immunol. 143:2714-2722 |
| rF-PSA | pT2078 | PSA | Hodge et al., 1995, Int. J. Cancer, 63:231-237 |
| rV-muc-1 | pT2068 | muc-1 | Gendler et al., 1990, J. Biol. Chem., 265:15286-15293 |

* rV indicates vaccinia recombinant
rF indicates fowlpox recombinant

15

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 23 -

What is claimed is:

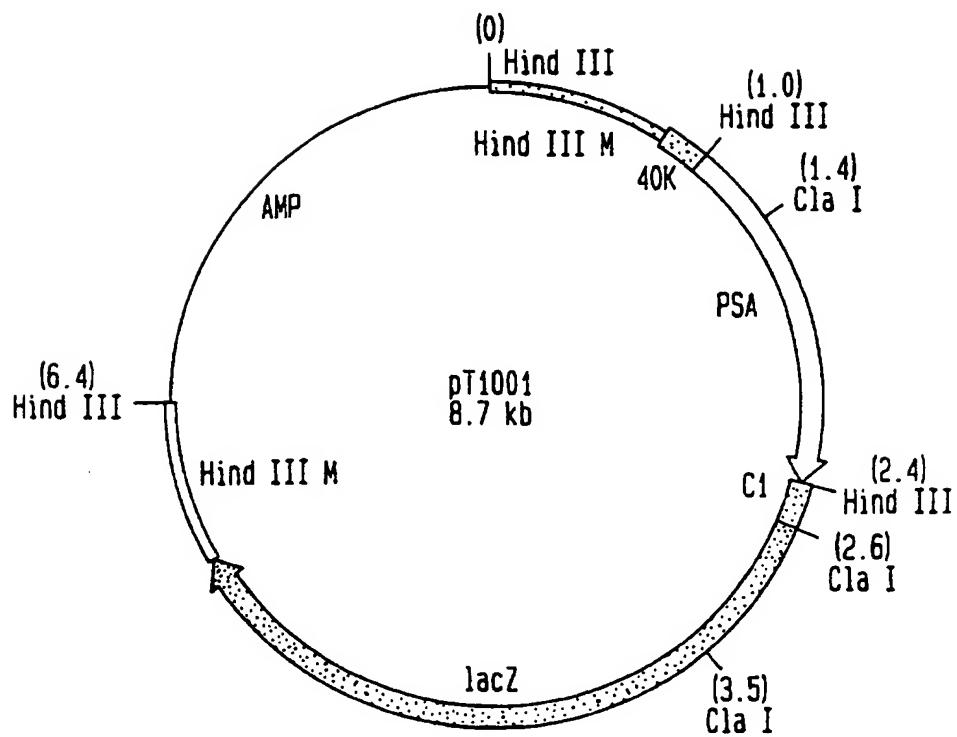
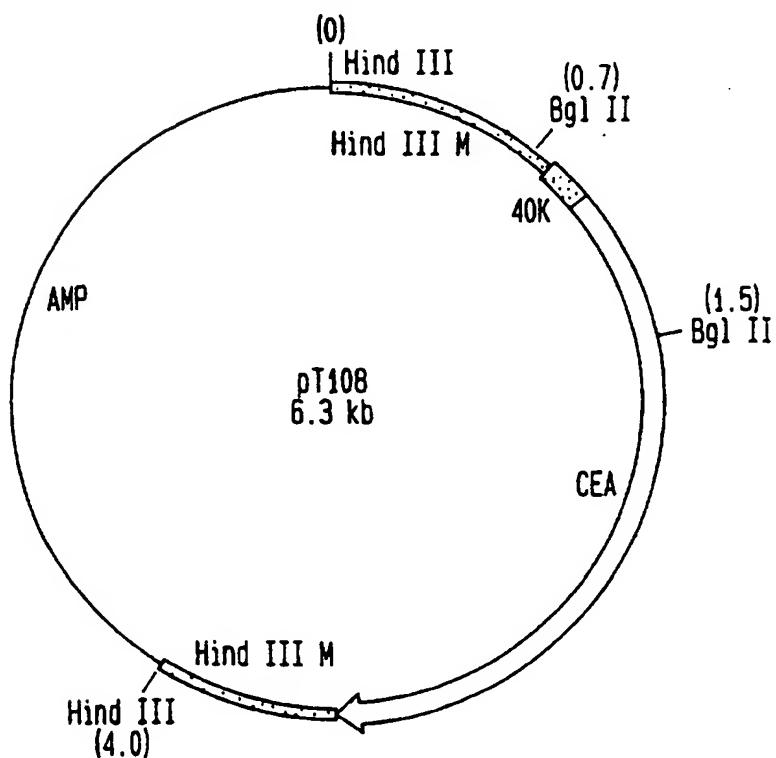
1. A recombinant pox virus selected from the group consisting of TBC-CEA, PROSTVAC, rV-B7.1, rF-B7.1, rF-CEA, rV-MUC-1 and rF-PSA.
2. The recombinant pox virus of claim 1, wherein the pox virus is TBC-CEA.
3. The recombinant pox virus of claim 1, wherein the pox virus is PROSTVAC.
4. The recombinant pox virus of claim 1, wherein the pox virus is rV-B7.1.
5. The recombinant pox virus of claim 1, wherein the pox virus is rF-B7.1.
6. A recombinant pox virus of claim 1, wherein the pox virus is rF-CEA.
7. A recombinant pox virus of claim 1, wherein the pox virus is rV-muc-1.
8. The recombinant pox virus of claim 1, wherein the pox virus is rF-PSA.
9. A method for treating a host having a tumor expressing carcinoembryonic antigen comprising contacting the host with an effective amount of the recombinant pox virus TBC-CEA or rF-CEA.

- 24 -

10. A method for treating a host having a tumor expressing prostate specific antigen comprising contacting the host with an effective amount of the recombinant pox virus PROSTVAC or rF-PSA.

11. A method for treating a host having a tumor expressing muc-1 antigen comprising contacting the host with an effective amount of the recombinant pox virus rV-muc-1 or rF-muc-1.

12. A method for treating a host having a tumor comprising contacting tumor cells of said host with an effective amount of the recombinant pox virus rV-B7.1 or rF-B7.1.

FIG. 1**FIG. 2**

rF. CCA

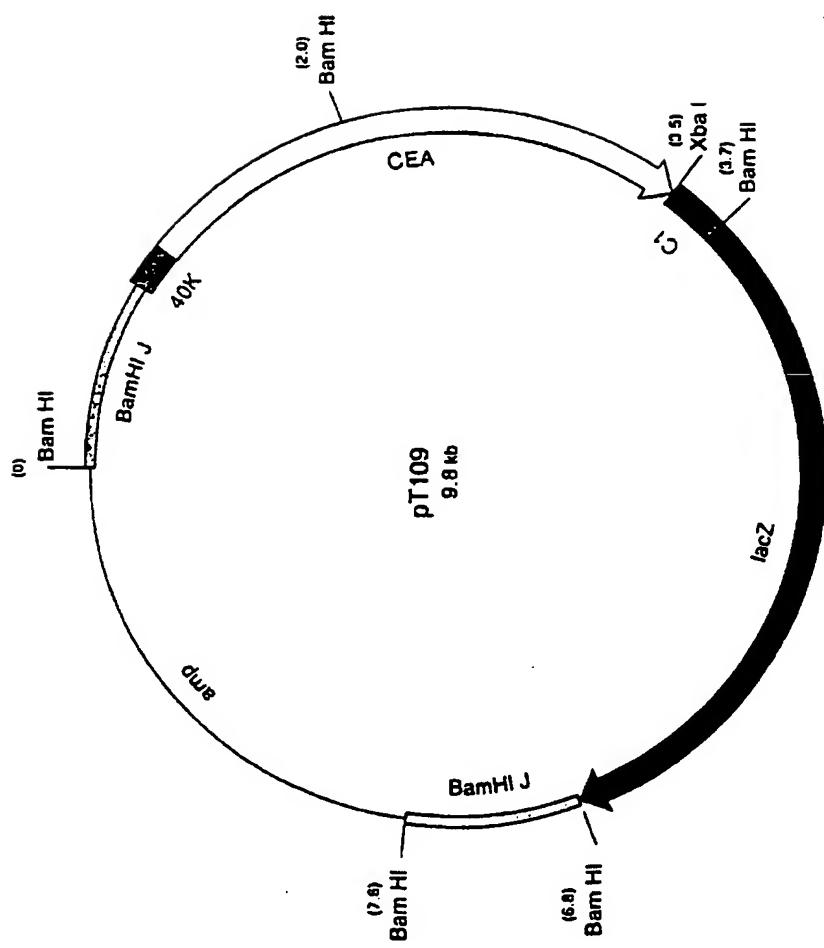


FIGURE 3

CV - P1.1

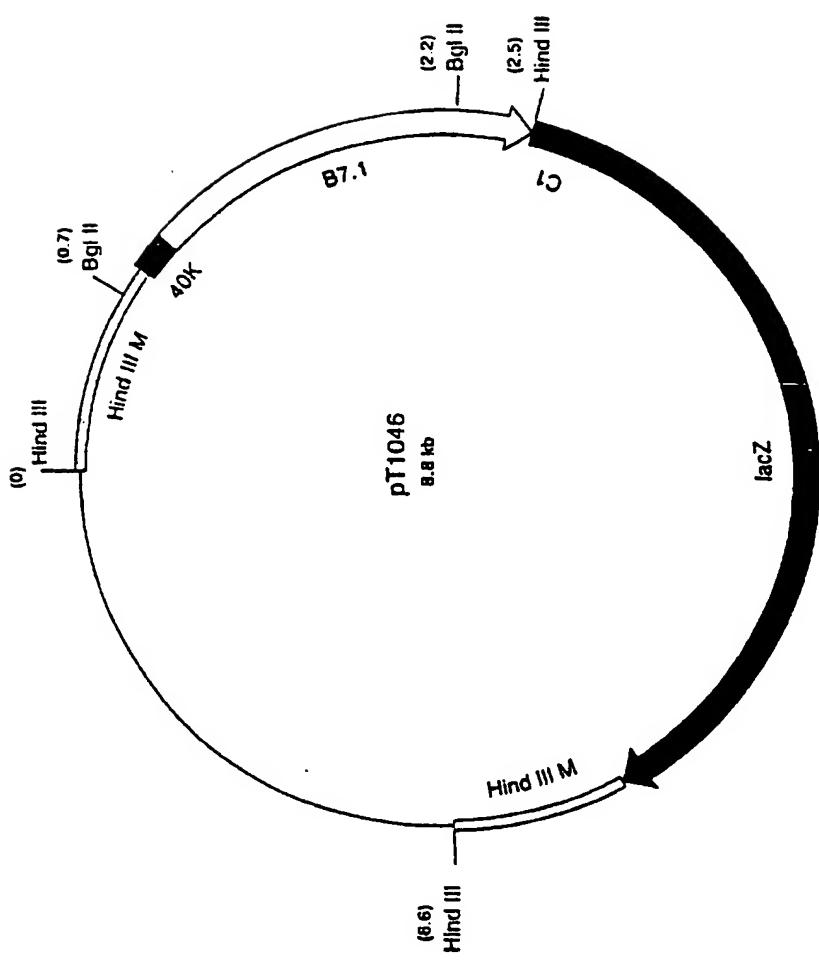


FIGURE 4

C 7.1.1

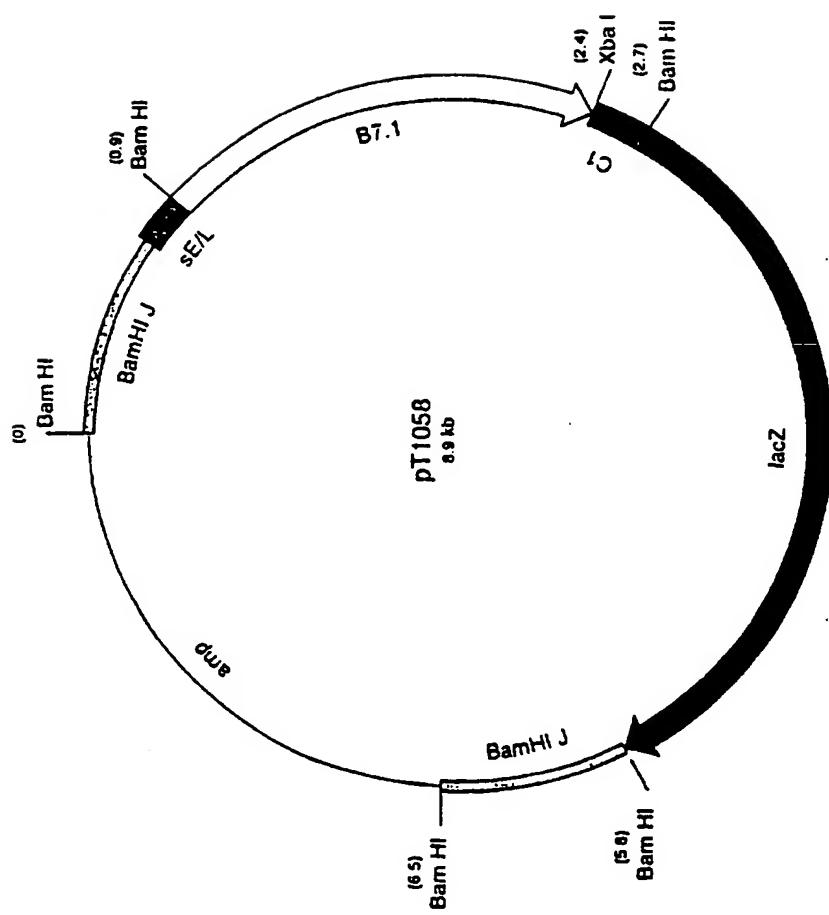


FIGURE 5

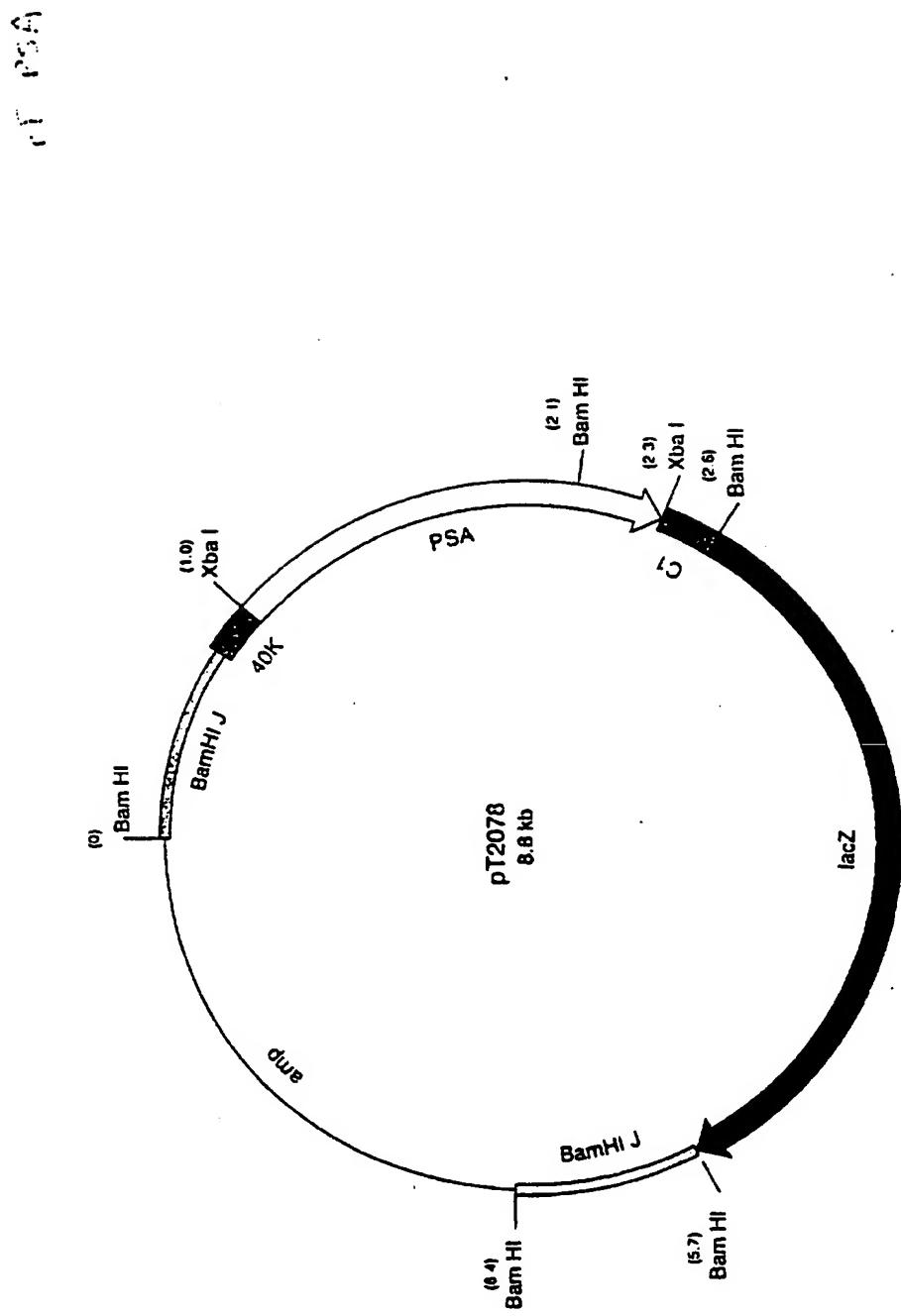
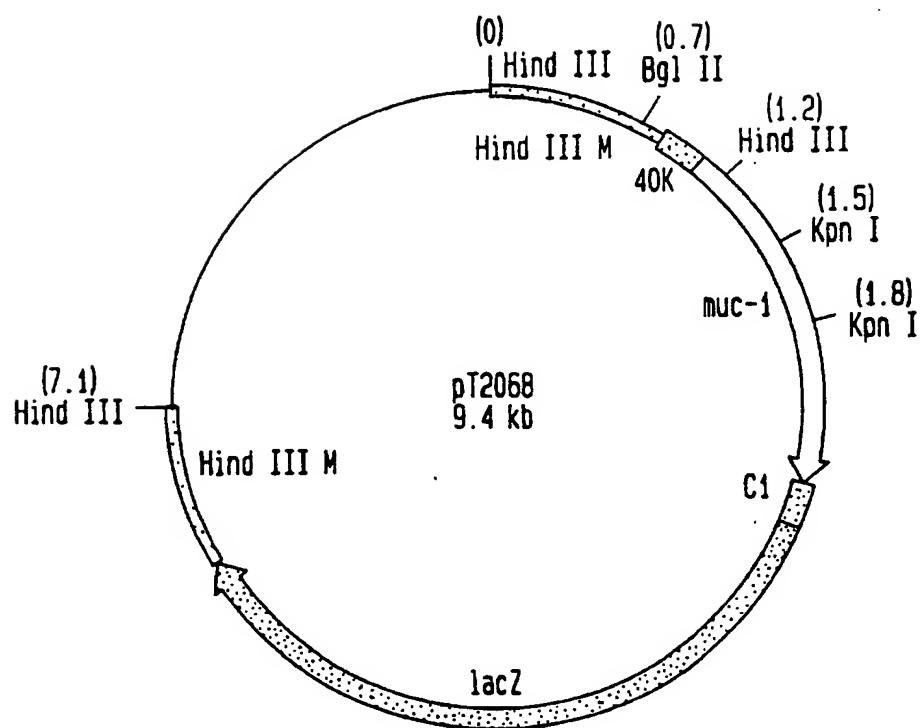


FIGURE 6

FIG. 7

INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/US 97/12203

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| P,X | WO 97 03203 A (THERION BIOLOGICS CORPORATION ET AL) 30 January 1997 see claims 1-16 see page 21, line 18 - page 22, line 20 --- | 1,3,8,10 |
| X | WO 96 11279 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 18 April 1996 see claims 1-27 --- | 1-12 |
| X | WO 96 10419 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 11 April 1996 see claims 1-13,16,17,19-26 --- | 1-12 |
| X | WO 94 16716 A (VIROGENETICS CORPORATION) 4 August 1994 see claims 1-20 --- | 1,2,4-6, 9,12 |
| | | -/- |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search

12 November 1997

Date of mailing of the international search report

27.11.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Siatou, E

INTERNATIONAL SEARCH REPORT

Int'l. Application No

PCT/US 97/12203

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| X | WO 92 19266 A (THE UNITED STATES OF AMERICA) 12 November 1992 see claims 1-10,16,17,23 --- | 1,2,9 |
| X | BALLOUL J - M ET AL: "RECOMBINANT MUC 1 VACCINIA VIRUS: A POTENTIAL VECTOR FOR IMMUNOTHERAPY OF BREAST CANCER" CELLULAR AND MOLECULAR BIOLOGY, vol. 40, no. Suppl. 1, 1 January 1994, pages 49-59, XP000561067 see page 51, left-hand column, line 26 - right-hand column, line 7 see page 57, right-hand column, line 1 - page 58, left-hand column, line 7 --- | 1,7,11 |
| X | IRVINE K ET AL: "COMPARISON OF A CEA-RECOMBINANT VACCINIA VIRUS, PURIFIED CEA, AND AN ANTI-IDIOTYPE ANTIBODY BEARING THE IMAGE OF A CEA EPITOPE IN THE TREATMENT AND PREVENTION OF CEA-EXPRESSING TUMORS" VACCINE RESEARCH, vol. 2, no. 2, 1 January 1993, pages 79-94, XP000577769 see abstract --- | 1,2,9 |
| X | KANTOR J ET AL: "ANTITUMOR ACTIVITY AND IMMUNE RESPONSES INDUCED BY A RECOMBINANT CARCINOEMBRYONIC ANTIGEN-VACCINIA VIRUS VACCINE" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 84, no. 14, 15 July 1992, pages 1084-1091, XP000577052 cited in the application see abstract --- | 1,2,9 |
| X | KANTOR J ET AL: "IMMUNOGENICITY AND SAFETY OF A RECOMBINANT VACCINIA VIRUS VACCINE EXPRESSING THE CARCINOEMBRYONIC ANTIGEN GENE IN A NONHUMAN PRIMATE" CANCER RESEARCH, vol. 52, 15 December 1992, pages 6917-6925, XP000605381 cited in the application see abstract --- | 1,2,9 |
| X | HODGE J W ET AL: "A RECOMBINANT VACCINIA VIRUS EXPRESSING HUMAN PROSTATE-SPECIFIC ANTIGEN (PSA): SAFETY AND IMMUNOGENICITY IN A NON-HUMAN PRIMATE" INTERNATIONAL JOURNAL OF CANCER, vol. 63, no. 2, 9 October 1995, pages 231-237, XP000604553 cited in the application see abstract --- | 1,3,10 |

-/-

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/12203

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| X | TSANG K Y ET AL: "GENERATION OF HUMAN CYTOTOXIC T CELLS SPECIFIC FOR HUMAN CARCINOEMBRYONIC ANTIGEN EPITOPES FROM PATIENTS IMMUNIZED WITH RECOMBINANT VACCINIA-CEA VACCINE" JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 87, no. 13, 5 July 1995, pages 982-990, XP000578019 see abstract ----- | 1,2,9 |

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. Application No

PCT/US 97/12203

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|--|------------------|---|--|
| WO 9703203 A | 30-01-97 | NONE | |
| WO 9611279 A | 18-04-96 | AU 3998295 A EP 0789774 A | 02-05-96 20-08-97 |
| WO 9610419 A | 11-04-96 | AU 3735395 A EP 0784483 A | 26-04-96 23-07-97 |
| WO 9416716 A | 04-08-94 | AU 6165294 A CA 2153336 A EP 0680331 A JP 9503902 T | 15-08-94 04-08-94 08-11-95 22-04-97 |
| WO 9219266 A | 12-11-92 | AU 674492 B AU 2006092 A CA 2102623 A EP 0584266 A JP 6508025 T | 02-01-97 21-12-92 07-11-92 02-03-94 14-09-94 |